PCT/AU03/01448

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WITNESS my hand this
Thirteenth day of November 2003

J.K.+C.

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AUSTRALIA

Patents Act 1990

Proteome Systems Intellectual Property Pty Ltd

PROVISIONAL SPECIFICATION

Invention Title:

Improved extraction of alkaline proteins

The invention is described in the following statement:

Field of the Invention

The present invention is in the field of analysing macromolecules, particularly, but not exclusively, with regard to the extraction of proteins for gel electrophoresis.

Background of the invention

In the field of analysing macromolecules, one-dimensional and two-dimensional gel electrophoresis methods have become standard tools for separating and visualising macromolecules. The highest resolution method for separating macromolecules is two-dimensional electrophoresis. Such two-dimensional electrophoresis usually involves sequential separations in a first dimension by isoelectric focusing and in a second dimension by SDS gel electrophoresis. Pre-treatment of samples for isoelectric focusing (IEF) involves solubilisation, denaturation and reduction to completely break the interactions between the proteins and remove non-protein sample components such as nucleic acids. Ideally to avoid protein losses, complete sample solubilisation is achieved in a single step, thus eliminating unnecessary handling.

Soluble protein samples can be readily taken up in the most commonly used IEF sample solution of 8M urea, 4% 3-[(3 cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS), 50-100 mM dithiothreitol (DTT) and 40 mM Tris. Typically, conventional protein extractions are done at alkaline pH (between pH 8 and 10), using Tris as the alkaline buffer. Convention wisdom suggests that alkaline pH favours protein solubility, because most proteins have acidic isoelectric points and thus are anions (and repel each other in solution) at alkaline pH. Furthermore, the alkaline pH is essential for reduction and alkylation cysteine, which has a pK of 8.3. Thus, the alkylation reaction occurs rapidly at pHs equal to or above 8.3.

The standard sample extraction solution IEF is not, however, ideal for many proteins and the challenge for two-dimension polyacrylamide gel electrophoresis (PAGE), particularly in the context of proteomics, is the solubilisation and separation of insoluble samples such as membrane and membrane-associated proteins and proteins from highly resistant tissues like

hair and skin. Methods for enhancing protein solubility which include introducing new reagents such as thiourea, sulfobetaine surfactants and tributyl phosphine into the IEF sample solution, have been described in, for example,

- Ravilloud, et al., Electrophoresis 1997, 18, 307-316;
- Chevallet et al., Electrophoresis 1998, 1901-1909;
- Herbert B R., Electrophoresis 1998, 19, 845-851.

A Gorg *et al* (Electrophoresis 2000, 21, 1037-1053) compared several methods of sample preparation for protein extraction which comprise cell disruption, precipitation in 20% TCA in acetone containing DTT overnight and solubilisation with sonication either in (i) in lysis buffer (ii) in thiourea lysis buffer or (iii) in hot SDS sample buffer.

More recently, methods of protein extraction which include reduction and alkylation of sample prior to the IEF step has been shown to further improve the resolution on two-dimensional maps. However, the problem of efficient and reproducible sample preparation of more insoluble samples is still unresolved. Further improvements in sample preparation for proteomic separation techniques will enable the resolution identification and study of a greater number of the more insoluble proteins.

Summary of the invention

In developing the present invention, the inventors have found that an alkaline extraction pH does not favour the solubility of alkaline proteins, because they are almost uncharged at the extraction solution pH. The inventors have sought to improve the extraction of alkaline proteins. The inventors have developed a method of treating a sample to extract proteins under acidic conditions. Preferably, the method of the invention provides increased solubilisation of alkaline proteins and greater elimination of cell wall artefacts from microorganisms. The method of the present invention does not require precipitation of the protein prior to solubilisation and preferably uses conditions which are relatively gentle. Preferably, for example, the method of the invention does not degrade proteins or remove sugars from a protein backbone during solubilisation.

Accordingly, in a first aspect the present invention provides a method of recovering macromolecules from a biological sample, the method comprising the steps of:

i. optionally disrupting the biological sample; and
ii. treating the biological sample with a reagent having a pH of about pH1 to about pH6 to solubilise at least one macromolecule in the biological sample.

Preferably, steps (i) and (ii) are performed simultaneously, sequentially or a combination thereof.

Preferably, the method further comprises the following step:

iii. precipitation and resolubilisation of the at least one solubilised macromolecule.

Preferably, the method further comprises the following step:

iv. reducing and alkylating the at least one resolubilised macromolecule.

In a second aspect the invention provides a method of recovering and resolving macromolecules in a biological sample, the method comprising the steps of:

i. optionally disrupting the biological sample;

ii. treating the biological sample with a reagent having a pH of about pH1 to about pH6 to solubilise at least one macromolecule in the biological sample; and

iii. resolving the at least one solubilised macromolecule present in the sample.

Preferably step (iii) is performed using proteomic techniques.

In a third aspect the invention provides a kit for recovering macromolecules in a biological sample, the kit comprising a reagent to solubilise at least one macromolecule in a biological sample, wherein the reagent has a pH of about pH 1 to about pH 6 and optionally comprising directions to recover a macromolecule in a biological sample, and optionally comprising directions to resolve a macromolecule in a biological sample.

In a fourth aspect the invention provides a macromolecule recovered by the method of the first aspect.

In a fifth aspect the invention provides a macromolecule recovered and resolved by the method of the second aspect.

The method of the present invention is particularly suitable for the recovery of proteins from cellular material and the like, preferably membrane and membrane associated proteins.

Accordingly, in a sixth aspect, the present invention provides a method for the recovery of a protein selected from membrane proteins, membrane-associated proteins, and tissue-associated proteins from cellular material, the method comprising contacting the cellular material with an extraction and/or resuspension agent having a pH in the range of about 1 to about 6.

The extraction and/or resuspension agent may be known composition but which has a pH in the range specified above.

In a seventh aspect, the invention provides an extraction and/or resuspension reagent having a pH in the range of about pH1 to about pH6.

Brief description of the figures

Figure 1. is a copy of a photographic representation of IPG gel strips comparing the extraction of B. Subtilis using an A. "old" and B. "new" reagent to solubilise the proteins.

Figure 2. is a copy of a photographic representation of IPG gel strips comparing the extraction of *S. Cerevisiae* using an A. "old" and B. "new" reagent to solubilise the proteins.

Figure 3. is a copy of a photographic representation of IPG gel strips showing increased alkaline protein yield.

Figure 4. is a copy of a photographic representation of IPG gel strips comparing the extraction of *E. Coli* using an A. "old" and B. "new" reagent to solubilise the proteins.

Figure 5. is a copy of a photographic representation of IPG gel strips comparing the extraction of *S. Cerevisiae* with four diffferent reagents having a pH of between pH 1 and pH 6.

Figure 6. is a copy of a photographic representation of IPG gel strips comparing the extraction of whole yeast cells using a A. reagent comprising citric acid, and B. reagent comprising Tris.

Figure 7. is a copy of a photographic representation of IPG gel strips comparing the extraction of *B. subtilis* using a A. reagent comprising citric acid, and B. reagent comprising Tris.

Detailed description of the invention

The present invention provides a method of recovering macromolecules from a biological sample, the method comprising the following steps:

i. optionally disrupting the biological sample; and
ii. treating the biological sample with a reagent having a pH of about pH1 to about pH6 to solubilise at least one macromolecule in the biological sample.

Preferably, steps (i) and (ii) are performed simultaneously, sequentially or a combination thereof.

As used herein, the term "simultaneously" means occurring or operating at the same time or concurrently. Accordingly, in one embodiment, steps (i) and (ii) are performed concurrently, ie., the sample is disrupted and treated with a reagent to solubilise at least one macromolecule concurrently.

As used herein, the term "sequentially" means occurring or following in succession. In one embodiment, step (i) is completed prior to the commencement of step (ii). ie., the sample is disrupted and thereafter the sample is treated to solubilise at least one macromolecule. Preferably, there are no intermediate steps between steps (i) and (ii).

In other embodiments steps (i) and (ii) can be performed sequentially and simultaneously. For example, steps (i) and (ii) can be performed partly at the same time. In one embodiment, the sample is partially disrupted prior to commencement of step (ii). In one embodiment, disruption of the sample is completed at the same time as the sample is treated to solubilise of at least one macromolecule.

Preferably, where steps (i) and (ii) are performed sequentially, the time between steps (i) and (ii) are minimised.

The risk of artefactual proteolysis (ie., proteolysis by the organisms own enzymes) is increased following cell disruption.

Preferably, steps (i) and (ii) are performed such that artefactual proteolysis or acid hydrolysis is minimised.

In one embodiment, the reagent has a pH of about pH 2 to about pH 5. In another embodiment, the reagent has a pH of about 3 to about pH 4.

The pH of a solution can be determined experimentally with a suitable pH colour indicator, or potentiometrically, with a pH meter (eg glass electrode). The pH of acid and base solution can also be approximately calculated from their molar concentrations.

As used herein the term "macromolecules" include, for example, proteins, glycoproteins, peptides and fragments thereof. Preferably, the macromolecules have an isoelectric point above about pH 7, more preferably above about pH 8, more preferably above about pH 8.2. Preferably, the macromolecule is an alkaline protein. By "alkaline protein" is meant a protein having an isoelectric point as predicted from the amino acid sequence of the protein or alternatively as determined empirically by isoelectric focussing or reverse phase chromatography. Preferred alkaline proteins are selected from the group consisting of alpha and beta chains of haemoglobin, immunoglobulins (eg., antibodies) and ribosomal proteins, membrane and membrane–associated proteins, and proteins found in highly resistant tissues eg. skin and hair. In a particularly preferred embodiment the alkaline protein is a membrane protein or a protein associated with a cellular fraction of a cell lysate that is relatively insoluble in aqueous solution containing 7M urea, 2M thiourea and 1% (w/w) C7 (pH8.0-8.5) or equivalent.

As used herein, the term "biological sample" refers to a sample comprising a macromolecule. Preferably, a biological sample is a cellular or tissue sample or culture thereof, such as, for example, a microbial sample (bacterial, yeast or fungal), a plant cell sample or seed, an animal cell or tissue sample including skin and hair samples and any other cellular material.

The term "disruption" refers to breaking apart or breakdown of a cellular or tissue sample, such as for example cell lysis, breakdown of tissue or breakdown of cell walls or membranes. Methods of disruption vary according

to the nature of the sample, and typically include autolysis (eg. by incubation with toluene), enzymatic lysis (eg. with lysozyme or β-glucanase), grinding, liquid nitrogen cooling, glass-bead beating, or sonication. Tissues can be disrupted in the presence of sample solubilisation buffer.

Preferably, the sample is sonicated.

It is understood that the biological sample can be disrupted prior to or at the same time as treatment of the biological sample to solubilise at least one macromolecule in the sample.

In one embodiment the biological sample is disrupted and treated concurrently. Preferably, the sample is sonicated in the reagent having a pH of about pH 1 to about pH 6.

Preferably, the reagent in which at least one macromolecule is solubilised comprises an aqueous solvent such as an acidic aqueous solvent selected from the group consisting of an organic acid solution, inorganic acid solution, acidic buffer, amino acid solution or a mixture thereof.

Preferably, an organic acid is selected from the group comprising acetic acid, ascorbic acid (~ pH 3-4), carboxylic acid eg benzoic acid (~ pH 2-3), polycarboxylic acid eg citric acid (~ pH 2-3) and derivatives and mixtures thereof.

In one embodiment, an inorganic acid is selected from the group comprising phosphoric acid (~ pH 2-3), and hydrochloric acid (~ pH 1) and derivatives and mixtures thereof.

Buffers are particularly useful in the present invention as the pH can be adjusted in the range of about pH 1 and about pH 6. One example of an acidic buffer is citrophospho buffer.

In one embodiment, the reagent comprises an amino acid, for example, aspartic acid or glutamic acid.

Preferably, the reagent further comprises a chaotropic agent (eg. urea, thiourea, or mixture thereof), or a detergent (eg ionic detergent, anionic detergent, non-ionic detergent etc).

Preparation of biological samples typically also comprises inactivation or removal of interfering substances, such as for example, proteolytic enzymes. Proteolytic enzymes can be inactivated by the addition of urea, thiourea, DTT, proteinase inhibitors, Tris base, acetone precipitation, or by boiling the sample in SDS buffer.

In one embodiment, a protease inhibitor is added to the reagent or biological sample.

In one embodiment, the method further comprises the following step:

iii. precipitation and resuspension of the at least one solubilised macromolecule.

Typically, precipitation is performed by addition of an organic solvent such as for example, methanol or acetone to the at least one solubilised macromolecule. Preferably, the solvent is added in a ratio of between 4 and 15 times the volume of the sample to be precipitated. In one embodiment, the organic solvent is at room temperature, or below room temperature. In one embodiment, the sample/solvent mix is allowed to precipitate for approximately 5 minutes to 15 hours. In a preferred embodiment, the precipitate is centrifuged at around 5000g for approximately 10 minutes.

In a preferred embodiment, the precipitate is resuspended in the reagent having a pH of about pH 1 to about pH 6.

According to a preferred method of the invention, the method further comprises the following step:

iv. reducing and alkylating the at least one resuspended macromolecule.

Preferably, reducing and alkylating the at least one resuspended macromolecule comprises treating the at least one resuspended macromolecule with one or more agents to reduce and alkylate one or more

macromolecules in the sample. Preferably, the one or more reducing or alkylating agents, are selected from dithiothreitol (DTT), Tri-n-butylphosphine (TBP), beta-mercaptoethanol, iodoacetamide, vinyl pyridine, acrylamide, iodoacetic acid or a mixture thereof.

In one embodiment, the resuspended macromolecule is reduced with Tri-n-butylphosphine (TBP), preferably 5mM TBP. In a preferred embodiment, the sample is reduced with Tri-n-butylphosphine (TBP), then alkylated with acrylamide, preferably 10mM acrylamide.

In a second aspect the invention provides a method of recovering and resolving macromolecules in a biological sample, the method comprising the steps of:

i. optionally disrupting the biological sample;
ii. treating the biological sample with a reagent having a pH of about pH1 to about pH 6 to solubilise at least one macromolecule in the biological sample; and
iii. resolving the at least one solubilised macromolecule present in the sample.

Preferably the macromolecule is resolved using proteomic techniques.

In one embodiment, the method of the second aspect further comprises the following step prior to step (iii):
precipitation and resuspension of the at least one solubilised macromolecule.

According to a preferred embodiment of the second aspect, the method further comprises the following step prior to step (iii):
reducing and alkylating the at least one solubilised and/or precipitated and suspended macromolecule.

Preferably, resolving the at least one macromolecule in step (iii) comprises any of the following proteomic techniques:
two-dimensional electrophoresis, one-dimensional electrophoresis, HPLC or liquid chromatography-mass spectrometry (LC-MS). In particular, mass spectrometry can be used to identify macromolecules.

In a preferred embodiment of the second aspect, the method further comprises the following steps:

iv. digesting the resolved macromolecules; and

v. identifying the digested macromolecules by mass-spectrometry.

Preferably, the macromolecules are digested by proteolytic enzymes.

In a third aspect the invention provides a kit for recovering macromolecules in a biological sample, the kit comprising a reagent to solubilise at least one macromolecule in a biological sample, wherein the reagent has a pH of about pH 1 to about pH 6 and optionally comprising directions to recover a macromolecule in a biological sample and optionally comprising directions to resolve a macromolecule in a biological sample.

In one embodiment, the kit comprises an additional agent such as any one or more of urea, thiourea, detergent, water or proteinase inhibitor.

In one embodiment, the reagent further comprises the additional agent(s).

In one embodiment, the kit further comprises an agent for disrupting the biological sample, eg., toluene. In a further embodiment, the kit comprises a reduction and alkylating solution.

In one embodiment, the kit comprises a means for resolving at least one macromolecule present in the biological sample. In one embodiment, the means for resolving at least one macromolecule comprises an electrophoresis gel.

In one aspect, the invention provides an extraction and/or resuspension reagent having a pH in the range of about pH1 to about pH6. Preferably, the reagent comprises an aqueous solvent such as an acidic aqueous solvent selected from the group consisting of an organic acid solution, inorganic acid solution, acidic buffer, amino acid solution or a mixture thereof.

Preferably, the reagent further comprises a chaotropic agent or detergent or mixture thereof.

Methods of the invention

This protocol can be used for the extraction of protein from any cell or tissue material for 2-D PAGE separation. The procedure detailed in this document uses yeast as a specific example of the steps required to prepare a sample.

Reagents

(1) Conventional Extraction/Resuspension Reagent

7M urea (*Sigma product, cat. no. U-6504*), 2M thiourea (*Sigma product, cat. no. T-7875*), 1% C7 (*Sigma product, cat. no. C-0856*), 40mM Tris (*Sigma product, cat. no. T-1503*).

weigh into a 15mL Falcon tube :
4.2 g of Urea
1.52g of thiourea
0.0484g of Tris(hydroxymethyl)methylamine
0.1g of C7 (detergent)
add milliQ water (18 MΩ.cm) to a final volume of 10mL

Dissolve the reagents by placing into the ultrasonic bath and sonicating for as short a time as possible. If the water in the ultrasonic bath is becoming too hot (above 30°C), add some ice. If the sample extraction solution is allowed to become too hot, the urea will degrade.

note: *this solution is also available in the Sigma (Sigma product, cat. no. C-0356).*

(2) New (citric acid) Resuspension Reagent

Weigh into a 15mL Falcon tube :
4.2 g of Urea
1.52g of thiourea
0.154g of citric acid (80mM final conc.)

0.1g of C7 (detergent)

add milliQ water (18 MΩ.cm) to a final volume of 10mL

dissolve the reagents by placing into the ultrasonic bath and sonicating for as short a time as possible. If the water in the ultrasonic bath is becoming too hot (above 30°C), add some ice. If the sample extraction solution is allowed to become too hot, the urea will degrade.

(3) Tributylphosphine (TBP) solution
200mM TBP (*Sigma product, cat. no. T-6918*)

note: complete this procedure in a fumehood!

Add 1mL of stock TBP to 19mL of 2-propanol (*Sigma product, cat. no.* 27,049-0) (flammable cabinet)

Flush the stock TBP and the 200mM TBP with helium for 1 minute.

note: this solution has a shelf life of 1 week when stored at 4°C.
note: this solution is also available from Sigma (Sigma product, cat. no T-7567).

(4) Acrylamide solution

1M acrylamide (*Sigma product, cat. no. A-9099*)

Weigh into a 15 mL Falcon tube:
0.71gacrylamide

add milliQ water (18 MΩ.cm) to a final volume of 10mL

mix to dissolve

(5) Dithiothreitol (DTT) solution

2M DTT *(Sigma product, cat. no. 150460)*

Weigh into a 15 mL Falcon tube:
3.09g dithiothreitol

add milliQ water (18 MΩ.cm) to a final volume of 10mL

mix to dissolve

Procedure

Weigh 1g dry yeast *(Sigma product YSC-2, Sacchararomyces cerevisiae Type II)* into a 50mL Falcon tube.
Note: wet tissue or cells will be approximately 10X heavier than lyophilised material, ie. 200mg wet weight will be required to obtain a similar extraction to 20mg of dry weight. The starting amounts of tissue can be varied, but the ratios of sample solution to tissue should always remain the same.

Record the yeast lot number.

Microbial sample preparation note: bacterial, yeast and fungal samples can be washed in milliQ water prior to sample prep. This is usually done to ensure any residual culture media is removed. Mammalian samples cannot be washed in water, as osmotic lysis will occur. Washing of mammallian cells or tissues must be done in isotonic buffer, such as phosphate buffered saline.
Wash the cells by adding to the yeast milliQ water (18 MΩ.cm) to a final volume of 50mL. Vortex to suspend the cells. Agitate the suspension on a rocker for 5 minutes.

Centrifuge the suspension at 2500g for 15 minutes at 4°C to pellet cellular material.

Discard the supernatant and repeat the wash according to steps 3 and 4.

Extract the protein from the yeast cells by sonicating the washed cells in 20 mL of resuspension reagent and 0.5 mL of TBP solution. The method for preparing

resuspension reagent and TBP solution are outlined in the reagents section of this protocol.

Sonicate the suspension with a high power ultrasonic probe (such as the Branson digital sonifier units models s-250d & s-450d) for 3 x 30 seconds (intensity 70%, duty cycle 100%). Chill the suspension on ice between bursts of sonication to ensure suspension spends a minimum of time at temperatures greater than 35°C.

Transfer the suspension to a 20mL centrifuge tube.

Centrifuge the suspension at 38000g for 20 minutes at 20°C to pellet cell debris.

Precipitate the supernatant at room temperature by the addition of 5 volumes of acetone. Allow the precipitation to continue for between 10 and 30 minutes. Centrifuge at 5000g for 10 minutes to recover the protein pellet. Discard the supernatant.

Reduction and Alkylation Procedure

Resuspend the precipitate in 10ml of (1) Conventional Extraction/Resuspension Reagent

Reduce and alkylate 10mL of alkaline protein solution by adding 250µL of TBP solution and 100µL of acrylamide solution. The final concentrations of TBP and acrylamide in the protein solution are 5mM and 10mM respectively. The method for preparing TBP solution and acrylamide solution are outlined in the reagents section of this protocol.

(This step should be completed in the fume hood. Remove the lid of the TBP solution and transfer a 250 µL aliquot of the TBP solution to the protein solution. Pass a gentle stream of helium through the TBP solution for 1 minute before capping the TBP solution. (If using the Sigma TBP ampoules *(Sigma product, cat. no T-7567)* transfer the required volume of TBP from the ampoule to the protein solution and dispose of the ampoule and remaining contents. The

ampoules are designed for single use.)

Stand the protein solution at room temperature for 90 minutes to allow complete reduction and alkylation of cysteine residues.

Quench the alkylation reaction by adding 0.05 mL of 2M DTT solution. The final concentration of DTT in the protein solution is 10mM.

Centrifuge the suspension at 21,000g for 5 minutes to pellet any undissolved material.

Aliquot the sample for frozen storage or proceed to IPG strip rehydration.

Results

The gels in Fig 6 are whole extracts of yeast. A was extracted using the new method including citric acid. B was extracted using the conventional method using Tris. Note the absence of horizontal streaking at the acidic end of the A gel. This streaking is caused by cell wall material which binds to proteins and causes poor focusing. In addition, the cell wall material binds to the detergent causing the highly stained smearing visible at the bottom left of the B gel.

The gels in Fig 7 are whole extracts of Bacillus subtilis, a gram positive bacteria. Gel A was extracted using the new method including citric acid. B was extracted using the conventional method using Tris. Note the absence of horizontal streaking at the acidic end of the A gel. This streaking is caused by cell wall material which binds to proteins and causes poor focusing.

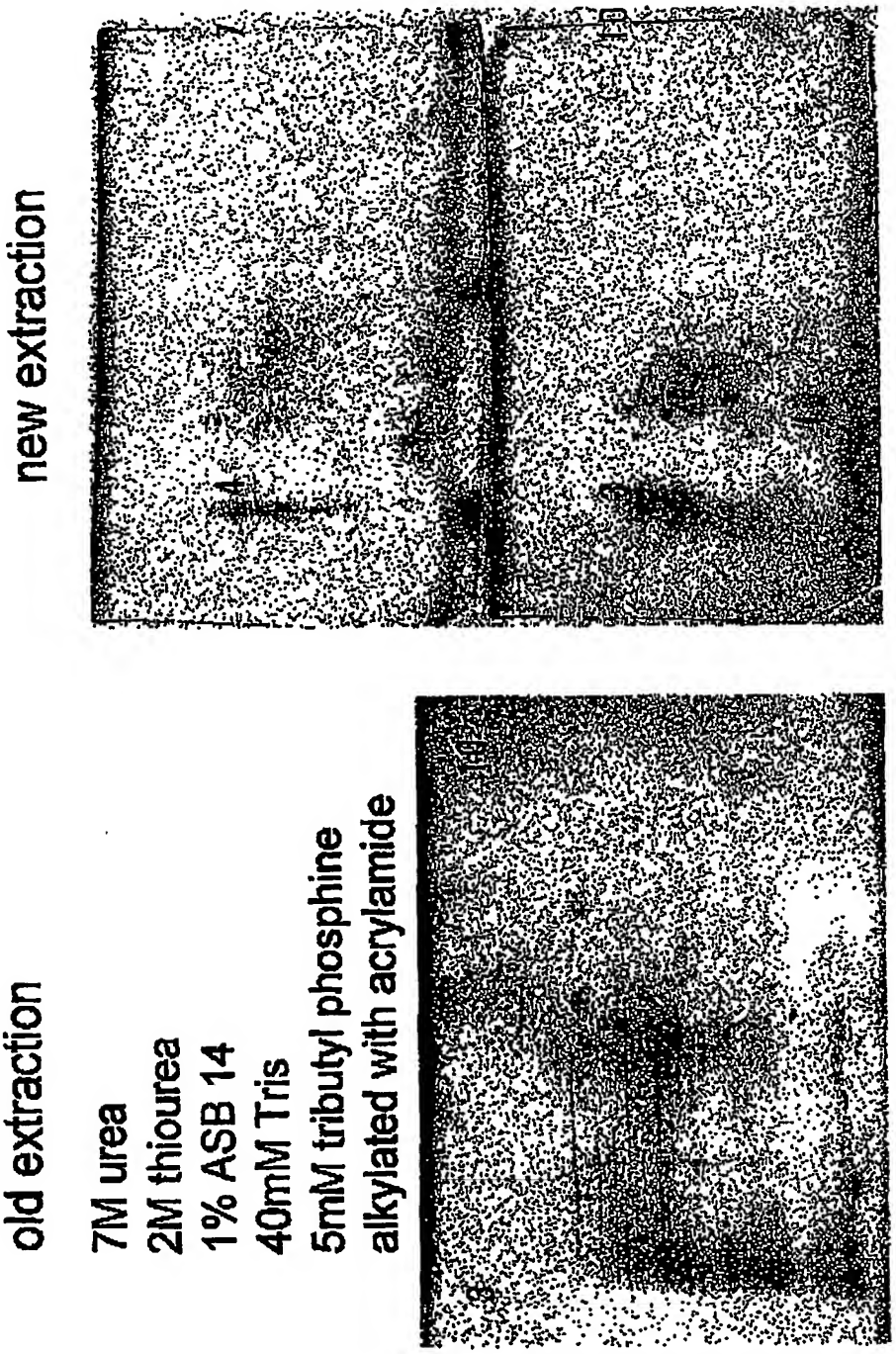
Further results are depicted in figures 1-5

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 4th day of November 2002

Proteome Systems Intellectual Property Pty Ltd
Patent Attorneys for the Applicant:

F B RICE & CO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1% ASB 14
40mM Tris
5mM tributyl phosphine
alkylated with acrylamide

FIGURE 1

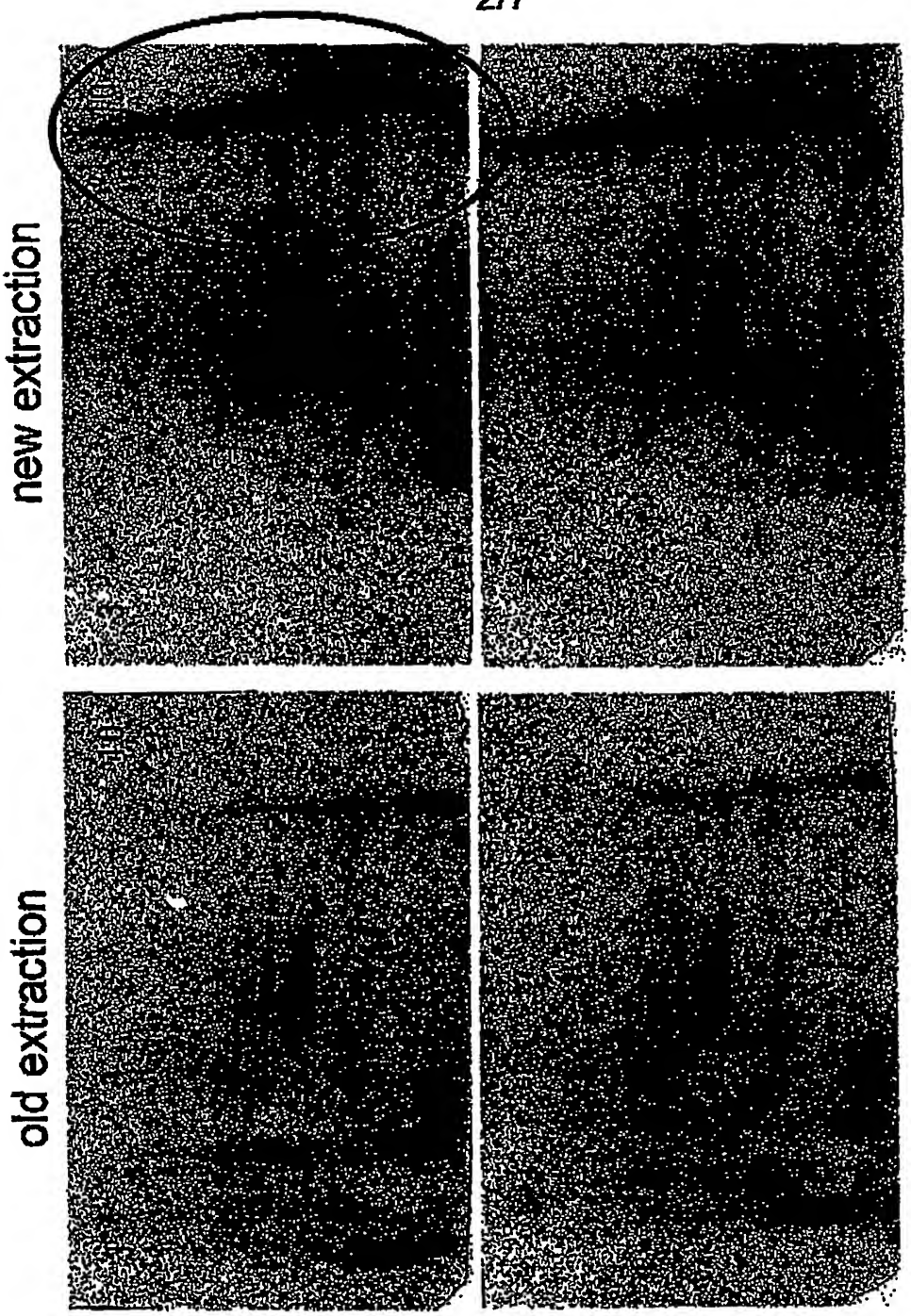


FIGURE 2

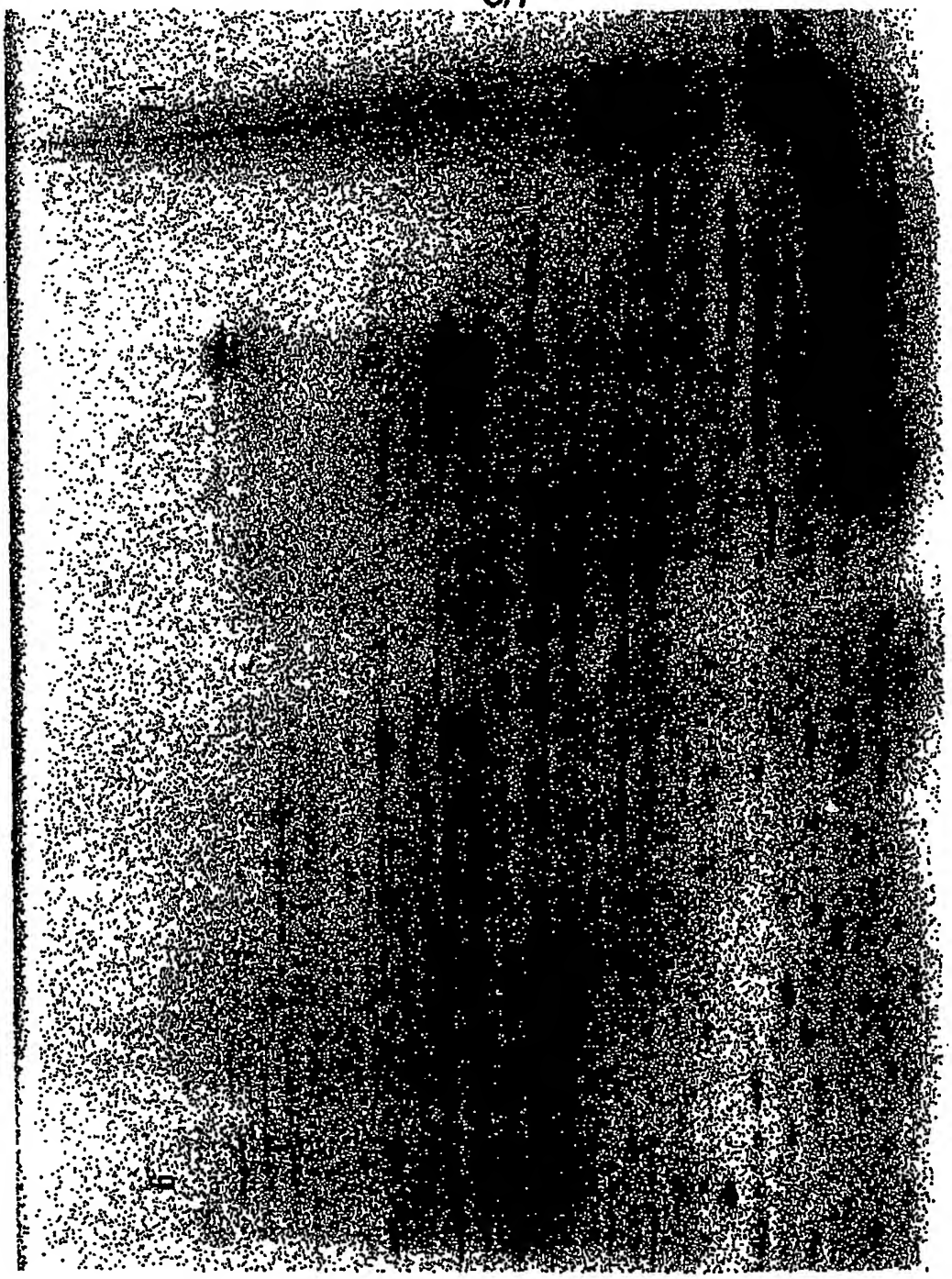


FIGURE 3

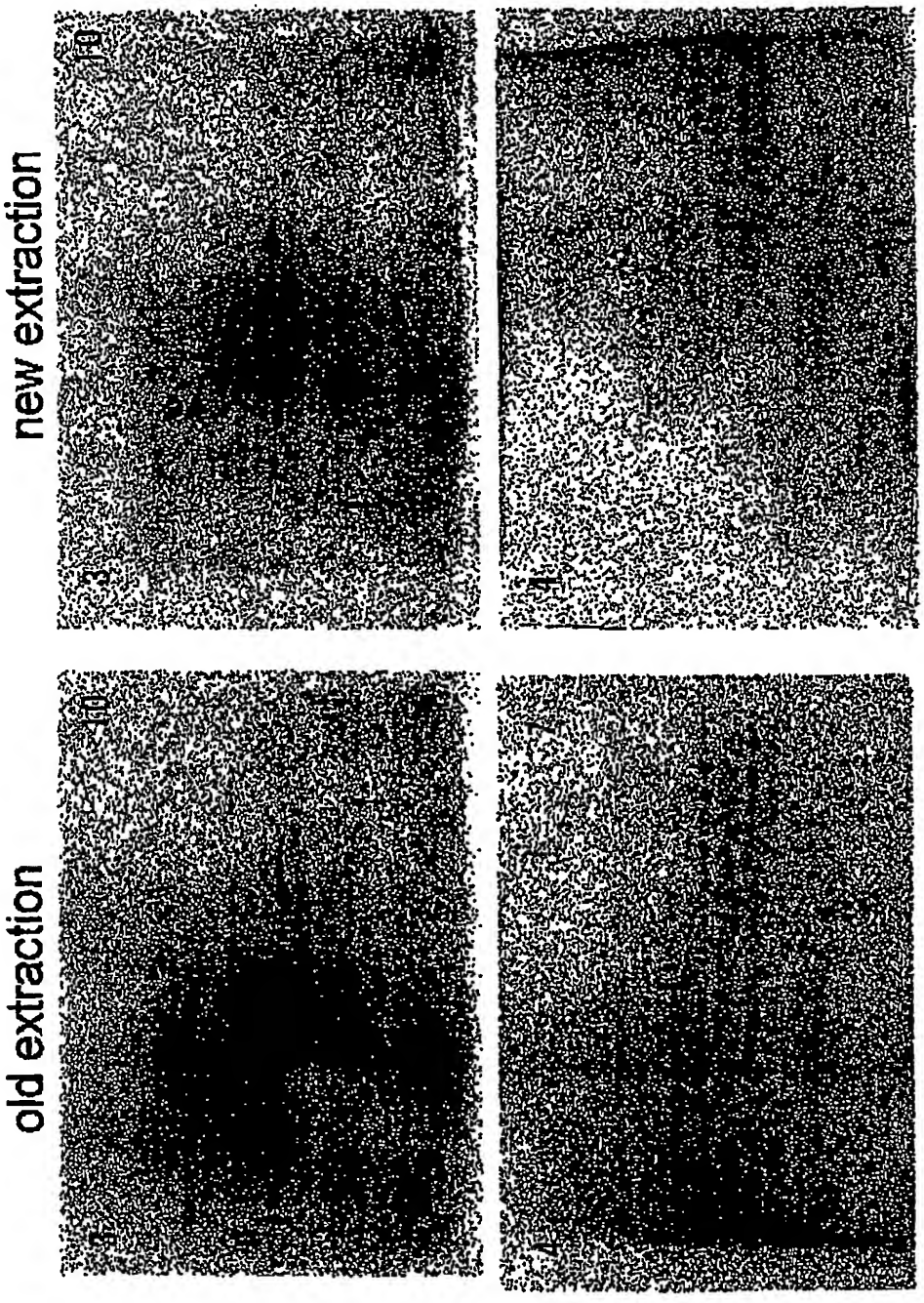


FIGURE 4

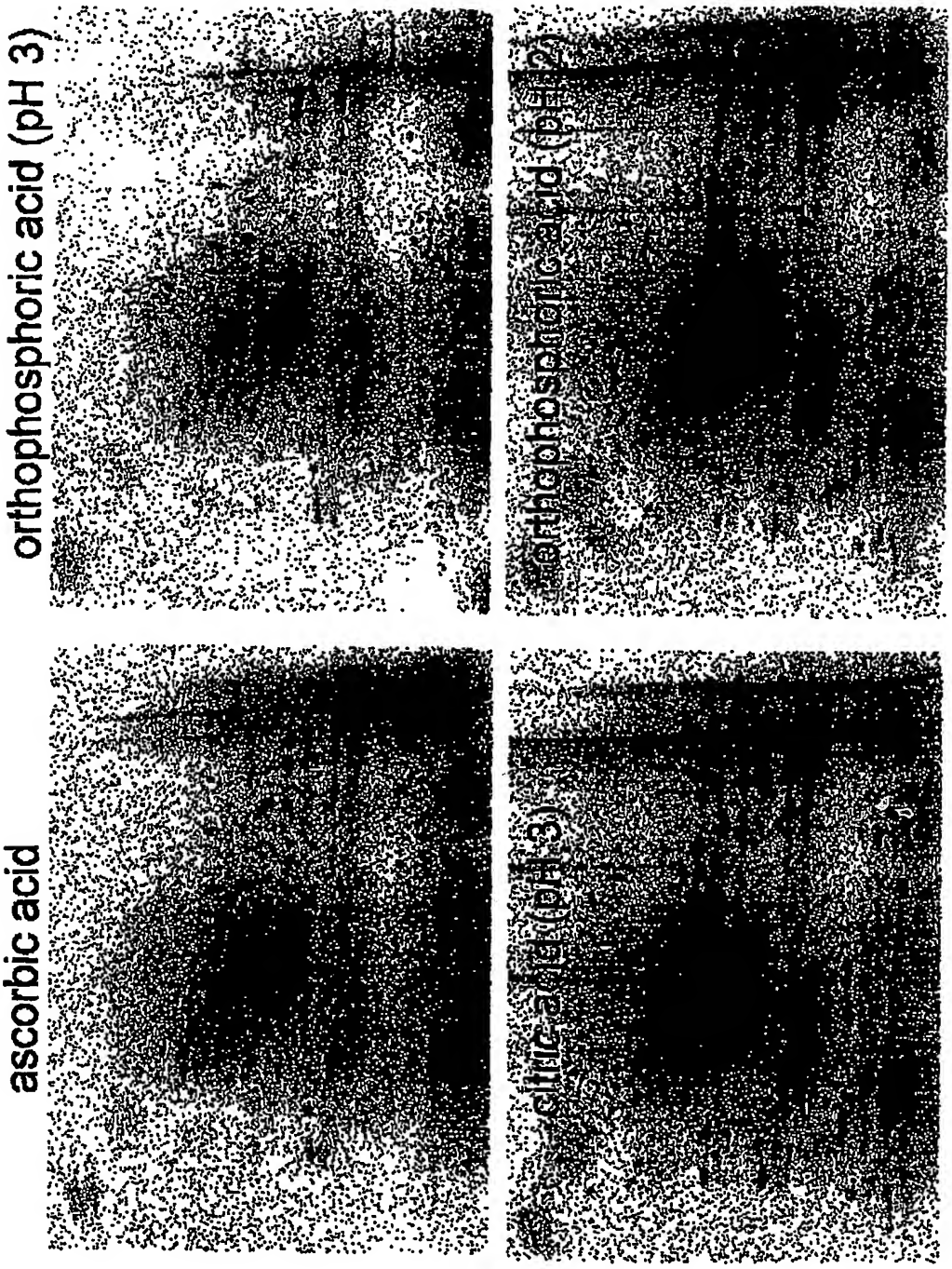
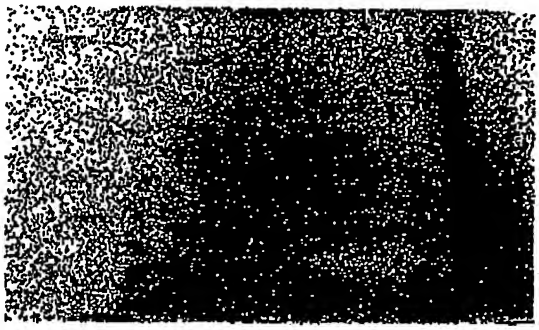

orthophosphoric acid (pH 3)
orthophosphoric acid (pH 3)
ascorbic acid
citric acid (pH 3)

FIGURE 5

Figure 6

A



B

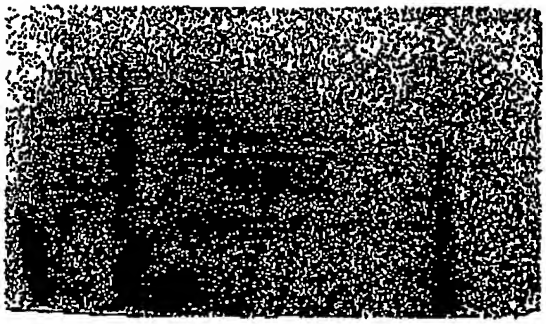
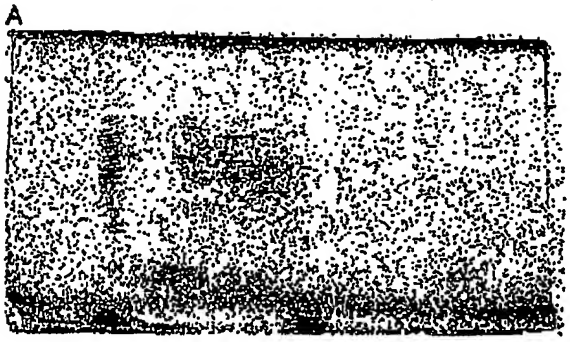


Figure 7.

A



B